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Liquid chromatography/mass spectrometry analysis of branched fructans produced *in vitro* with ^{13}C-labeled substrates

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RATIONALE: Fructans are carbohydrates predominantly based on fructose which are generally considered to be soluble dietary fibers with health-promoting properties. It is known that the nutritional properties of fructans are affected by their structure. This study focused on structural determination of branched fructans, as the most important dietary fructans are branched graminan-type fructans.
METHODS: Branched fructans were synthesized enzymatically by incubation of a heterologously expressed sucrose: fructan 6-fructosyltransferase (6-SFT) from *Pachysandra terminalis* with native or ^{13}C-labeled substrates. Liquid chromatography/mass spectrometry (LC/MS) was used for the structural identification of branched fructans. The MS^2 fragmentation of these compounds is described for the first time. Analytes were charged by electrospray ionization in negative mode and a quadrupole mass analyzer was used for MS^2 analysis.
RESULTS: The MS^2 fragmentation patterns of branched and linear fructans were shown to differ and distinctive ion formation allowed differentiation between all branched fructan isomers formed. *P. terminalis* 6-SFT preferred extending the existing fructan branch rather than creating a new branch.
CONCLUSIONS: The MS^2 fragmentation patterns described in the current paper now allow rapid screening of large sample sets for the presence of branched, graminan-type fructans. Furthermore, the data enables the characterization of fructan-metabolizing enzymes by identification of the fructan structures produced by *in vitro* reactions as described here for *P. terminalis* 6-SFT. Copyright © 2014 John Wiley & Sons, Ltd.

Fructans are fructose based carbohydrates containing no or one glucose unit per molecule.[1] They accumulate in about 15% of all flowering plants, and they are found in many economically important crops such as wheat,[2] barley,[3] and perennial ryegrass.[4] Fructan synthesis starts by the addition of fructose to a primary hydroxyl group of sucrose. Hence, three trisaccharides can be formed, i.e. 1-kestotriose (1-kestose), 6-kestotriose (6-kestose) and 6G-kestotriose (neokestose), that form the template for further elongation. The resulting fructan structures can be divided into five classes based on their trisaccharide core and on the type of fructose-fructosyl linkage. Inulin-type fructans are linear structures with almost only β(2-1) linkages. They are based on 1-kestotriose and have been studied intensively because of their health-promoting effects.[5] Levans have a linear structure but are based on 6-kestotriose and contain (almost) only β(2-6) fructose-fructosyl linkages. Neofructans are synthesized starting from 6G-kestotriose. Neo-inulins predominantly contain β(2-1) linkages whereas neolevans are neofructans with mainly β(2-6) linkages. Graminans are branched fructans with both β(2-1) and β(2-6) fructose-fructosyl linkages.[6] It is believed that graminans are the major fructan type in the vegetative tissues of most cereals.[7] Yet, both fructan concentration and structure may vary between tissue types and during the growing season.[8] Several previous studies have correlated fructan structure to apparent health benefits[9,10] and this is true of other types of dietary fiber[11] as well. However, the traditional methods for the analysis of fructans are laborious and complex. The structural identification of graminan is complicated by the fact that for each degree of polymerization (DP), various graminan isomers can be formed. Thus, as the molecules increase in molecular weight the number of possible structures grows exponentially.

Traditionally, methylation analysis in combination with gas chromatography coupled to mass spectrometry (GC/MS) has been widely used for fructan structural characterization.[12–14] Fructans are partly methylated, then hydrolyzed and the products are reacted to form alditol acetates. The relative proportions of the different fructan building blocks are revealed by GC/MS. In this way, an average composition of all fructans present in the sample can be obtained. If

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the methylation steps are preceded by a separation step, information on individual fructan structures can be obtained but only the structure of low DP fructans (DP <5) can be determined unambiguously.[12] Larger fructans are often hydrolyzed by specific enzymes and analyzed with GC/MS to obtain more structural information of the original compounds.[12] This approach is very time-consuming and inappropriate for large sample sets or for the study of many fructan structures. Nuclear magnetic resonance (NMR) can be used to study fructan structures and has the advantage that no derivatization step is required.[15] However, this method only gives an average composition of all fructan structures present in the sample unless it is preceded by a laborious purification to produce fractions of individual fructan structures. More recently, Suzuki *et al*. used a combination of direct infusion electrospray ionization mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to study the incorporation of glucose into fructans during fructan synthesis in asparagus tissue.[16] Harrison *et al*. reported the use of liquid chromatography coupled to mass spectrometry (LC/MS) to identify linear fructan structures.[17] They were able to separate inulin- and levan-type fructans and mapped their MS fragmentation patterns. The use of ^{13}C-labeled substrates and specific fructan-synthesizing enzymes allowed the synthesis of fructan structures that are not commercially available, which were used to understand their MS^2 fragmentation. Once this fragmentation pattern was established, it could be used to screen large sample sets for their presence without the need for fructan standards nor a preceding fructan purification step.[17] We hypothesize that LC/MS is suitable for the identification of branched fructans. The aim of the present study was to enzymatically synthesize branched, graminan-type fructans and to elucidate their MS^2 fragmentation patterns. To this end, a heterologous sucrose:fructan 6-fructosyltransferase (6-SFT) from *Pachysandra terminalis* was incubated with 1-kestotriose and either native sucrose or ^{13}C-labeled sucrose. In the latter case, the fructose unit of sucrose was either universally labeled with ^{13}C or labeled with ^{13}C at the first position only. The 6-SFT used transfers a fructose unit from sucrose to 1-kestotriose (or another fructan acceptor) and creates a β(2-6) fructose-fructosyl linkage.

LC/MS and LC/MS^2 analyses were used to identify the molecular structures formed during the enzymatic synthesis. The following workflow was applied:

(1) Identification of the molecular ions of the fructan present in the LC/MS analyses of the reaction mixture from the enzymatic synthesis. Comparison of the traces from the syntheses using unlabeled and ^{13}C-labeled substrates allowed the confirmation of the DP number and the number of fructose units that were incorporated.
(2) Collision-induced dissociation (CID)-MS^2 analysis of the major fructan isomers present.
(3) Higher energy collisional dissociation (HCD)-MS^2 analysis of the major fructan isomers present.
(4) Structural identification of the fructan isomers present by comparison of the MS^2 spectra from the unlabeled and the labeled isomers and with reference to the previous literature.

EXPERIMENTAL

Materials

All chemicals, solvents and reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade unless specified otherwise. Universally labeled sucrose or [UL-$^{13}C_6$ fru] sucrose (β-D-[UL-$^{13}C_6$] fructofuranosyl α-D-glucopyranoside) and [1-$^{13}C_1$ fru] sucrose (β-D-[1-$^{13}C_1$] fructofuranosyl α-D-glucopyranoside) were purchased from Campro Scientific (Veenendaal, The Netherlands). The fructose unit of [UL-$^{13}C_6$ fru] sucrose is composed of ^{13}C isotopes only. The fructose unit of [1-$^{13}C_1$ fru] sucrose contains a ^{13}C isotope only on the first carbon. 1-Kestotriose, 1,1-kestotetraose and 1,1,1-kestopentaose were purchased from Megazyme (Bray, Ireland). 6-Kestotriose was a generous gift from Dr. Iizuka[18] and 6G-kestotriose was purified from a *Xanthophyllomyces dendrorhous* culture broth.[19] 6-SFT from *Pachysandra terminalis* was obtained by heterologous expression in *Pichia pastoris*,[20] and was donated by Prof. W. Van den Ende.

Methods

Enzymatic synthesis of branched fructans

1-Kestotriose (300 mmol/L) was incubated with 6-SFT and either native sucrose or ^{13}C-labeled sucrose (300 mmol/L) at 20°C in 500 or 1000 µL sodium acetate buffer (100 mmol/L, pH 5.5) containing 0.02% (w/v) NaN_3. Reaction conditions were based on those described by Van den Ende *et al*.[20] Fructan formation was monitored with high-performance anion-exchange chromatography (HPAEC)[19] and a HPAEC profile of the fructan formation is shown in Supplementary Fig. S6 (see Supporting Information). Once at least 20% 1-kestotriose had been converted, 6-SFT was inactivated by

Table 1. Overview of the major DP 4, DP 5 and DP 6 oligosaccharides formed by 6-SFT. Retention time (t_r) and *m/z* values of the unlabeled molecular ions $[M–H]^-$ are provided together with the major CID MS^2 fragment ions formed starting from the unlabeled compound

Fructan	t_r (min)	*m/z* $[M–H]^-$	*m/z* CID MS^2 fragments
1&6-KT	2.4	665.21	485.15; 503.16
1,6-KT	3.7	665.21	341.11; 485.15; 503.16
1&6,6-KP	2.7	827.27	503.16; 647.20; 665.21
1,6&6-KP	2.7	827.27	503.16; 647.20; 665.21
1,6,6-KP	3.5	827.27	323.10; 341.11; 383.12; 503.16; 647.20; 665. 21; 707.22
6&1,6,6-KH	2.9	989.32	(*)
1,6&6,6-KH	3.1	989.32	665.21; 809.26; 827.27
1&6,6,6-KH	3.4	989.32	383.12; 485.15; 503.16; 545.17; 665.21; 809.26; 827.27
1,6,6,6-KH	4.1	989.32	(*)

(*) The major fragment MS^2 ions were not listed for 6&1,6,6-KH and 1,6,6,6-KH because the signal-to-noise ratio was low which made it impossible to make a comprehensive list of all ions formed.

incubating the reaction mixture at 100°C for 10 min after which it was filtered (0.2 µm) and 1000× diluted. The final reaction mixture contained at least 90 mmol/L glucose. Based on the detector response of 1,1-kestotetraose, the concentration of the formed DP 4 fructans was estimated to be at least 75 mmol/L.

Liquid chromatography/mass spectrometry

The fructan separation was performed using two trifunctional C18 alkyl columns (Acquity UPLC HSS T3, 100 × 2.1 mm, 1.8 µm particle size, Waters) placed in series. They were eluted isocratically with an 0.1% aqueous solution of formic acid as mobile phase for 15 min at a flow rate of 0.35 mL/min.[21] A 5 µL injection was used and the first 30 s of the analysis was diverted to waste. An orbitrap mass spectrometer (Orbitrap Fusion Tribrid; Thermo Scientific) was used as detector with data being acquired over a mass range of 500 to 2000 *m/z*. A heated electrospray ionization source was used at a temperature of 315°C in negative ion mode. For MS^2 analysis, the ions of interest were selected by the quadrupole using a window of 0.5 *m/z*. These ions were fragmented either by CID (30% collision energy) or by HCD (25% collision energy). Data were collected in centroid mode and analyzed with Xcalibur 3.0 software.

Mass spectral interpretation

The nomenclature of Domon and Costello[22] was used for the designation of the fructan fragments formed. For linear fructans, fragments containing the glucose unit are labeled as A_i, B_i and C_i, depending on the cleavage site relative to the glycosidic bond. The subscript i represents the number of the glycosidic bond cleaved, counting from the glucose end. Fragments in which the

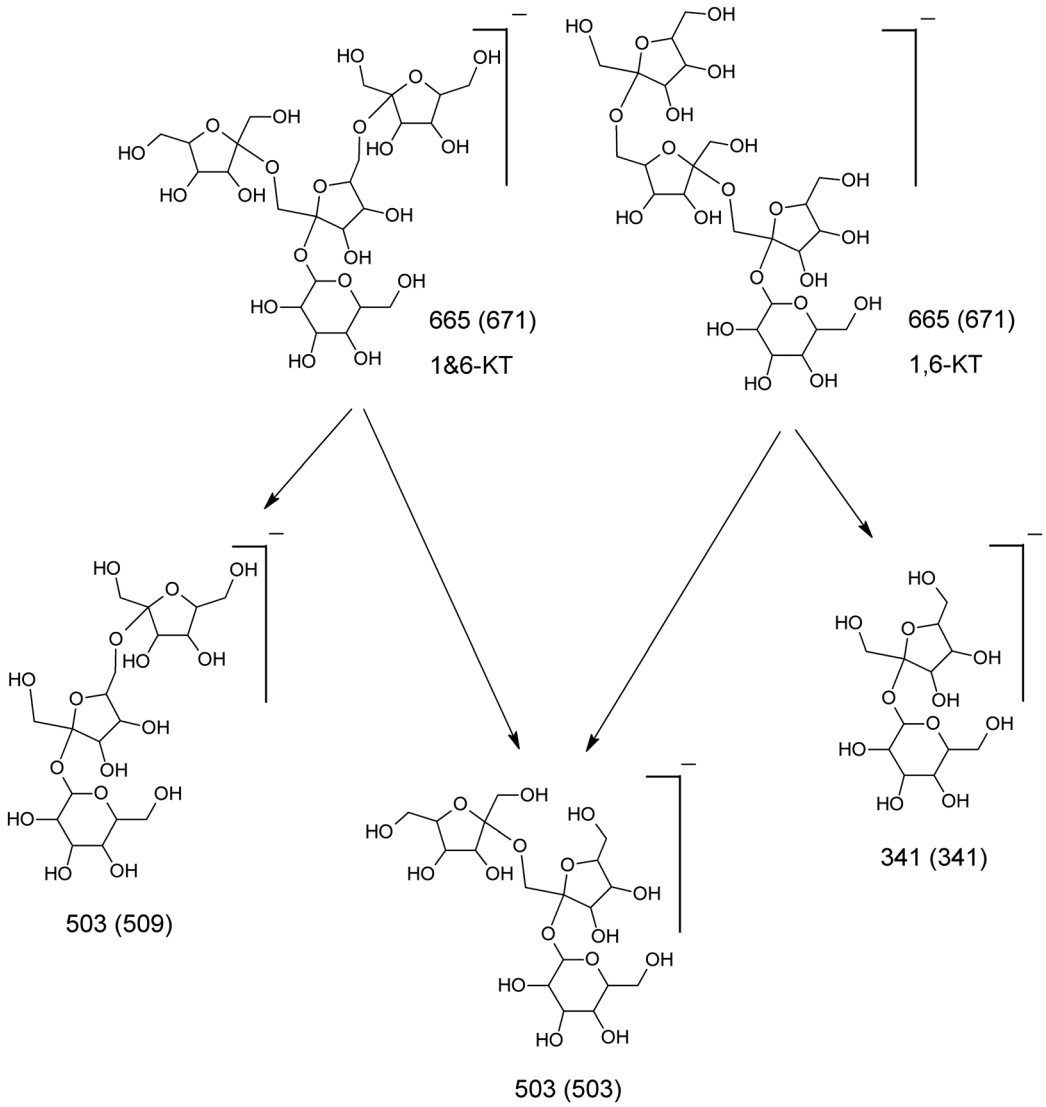

Figure 1. Molecular structures of 1&6-kestotetraose (1&6-KT) and 1,6-kestotetroase (1,6-KT) and their specific fragment ions formed in MS^2. The *m/z* values are indicated below the structural formula with between parentheses the *m/z* values of the corresponding [$UL^{13}C_6$ fru]-labeled species.

glucose unit was lost are designated as X_j, Y_j and Z_j, depending on the cleavage site relative to the glycosidic bond, and the j index counting starts from the intact fructose end. The letters A and X are used to designate fragments formed by cross ring cleavage, C and Y designate ions formed by breakage of the glycosidic bond with retention of water, whereas Z and B ions are formed by glycosidic bond cleavage involving a loss of water.[22] For branched fructans, fragments with both a terminal glucose and a terminal fructose are labeled with A_i, B_i and C_i, with an additional Greek letter is placed in subscript when the fragmentation occurs in a branch and α, β and γ represent the branch with the highest, the second highest and third highest molecular weight, respectively. The largest unbranched part containing glucose is called the core unit and its fragments receive no subscript Greek letter. In case this assignment of the core unit is ambiguous (when two potential core units with equal molecular weight are present), the portion with the most β(2-1) linkages is designated as the core unit.

RESULTS AND DISCUSSION

Enzymatic synthesis of branched fructans

Branched fructans containing both β(2-1) and β(2-6) linkages were produced *in vitro* with a heterologously expressed 6-SFT from *Pachysandra terminalis*. The fructan synthesis was monitored using HPAEC (Supplementary Fig. S6, see Supporting Information). Once the reaction was complete the reaction mixture was analyzed by LC/MS and LC/MS^2. Authentic standards were used to confirm the identity of some of the low molecular weight fructans (Supplementary Fig. S7, see Supporting Information). Comparison of the fragmentation patterns in the MS^2 spectra of the native and ^{13}C-labeled fructans allowed the identification of the remaining DP 4, DP 5 and DP 6 structures (summarized in Table 1). By far the majority of the product of the reaction were DP 4 fructans, with the concentrations of higher DP fructans decreasing with size, i.e. the concentration of DP 4 > DP 5 > DP 6 (Supplementary Table S1, see Supporting information) and the concentrations of higher molecular weight fructans (DP >6) were too low to allow structural identification. 1-Kestotriose and sucrose were the reaction substrates but a small amount of 6G-kestotriose was seen to be present (Supplementary Fig. S1(a), see Supporting Information). HPAEC analysis showed that this 6G-kestotriose was already present at the start of the reaction and not accumulated thereafter (Supplementary Fig. S6, see Supporting Information). If 6G-kestotriose had been formed during the reaction, [UL-$^{13}C_6$ fru] would have been incorporated in the newly formed 6G-kestotriose. However, although native 6G-kestotriose (*m/z* 503) was clearly present (Supplementary Fig. S1(a), see Supporting Information), no labeled 6G-kestotriose was observed in the extracted ion chromatogram (EIC) for 509 and 515, the *m/z* values of DP 3 fructans with one or two UL-$^{13}C_6$ fructose units, respectively (Supplementary Figs. S1(b) and S1(c), see Supporting Information). Hence, *P. terminalis* 6-SFT appears to have no fructan:fructan 6G-fructosyltransferase (6G-FFT) side activity. Labeled 6-kestotriose was present in small amounts (as shown by the peak in the EIC trace for *m/z* 515), together with minor amounts of labeled 1-kestotriose. These results are in accordance with those of Van den Ende *et al*.[20] who observed that 6-SFT from *P. terminalis* has an inherent 6-SST (6-sucrose: sucrose fructosyltransferase) activity and a 1-SST (1-sucrose: sucrose fructosyltransferase) side activity. *P. terminalis* 6-SFT is known to prefer 1-kestotriose over sucrose as a fructose acceptor and, therefore, it preferentially produces graminans, rather than levan-type fructans.[20]

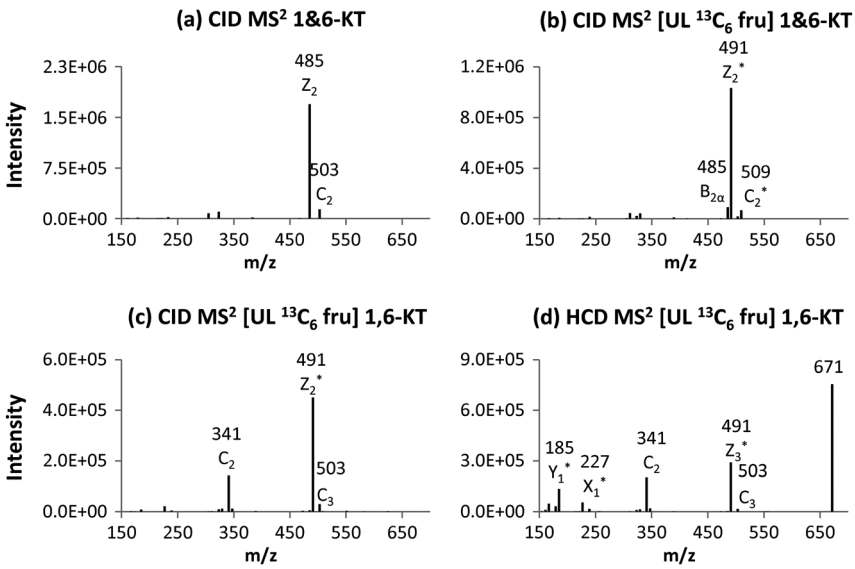

Figure 2. Negative electrospray MS^2 spectra of the main DP 4 fructans formed by 6-SFT. (a) CID MS^2 (*m/z* 665) of unlabeled 1&6-kestotetraose. (b) CID MS^2 (*m/z* 671) of [UL$^{13}C_6$ fru] 1&6-kestotetraose. (c) CID MS^2 (*m/z* 671) of [UL$^{13}C_6$ fru] 1,6-kestotetraose. (d) HCD MS^2 (*m/z* 671) of [UL$^{13}C_6$ fru] 1,6-kestotetraose.

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Fragmentation of DP 4 fructans

6-SFT can potentially synthesize two DP 4 fructans or kestotetraoses (KT) when 1-kestotriose acts as fructose acceptor. The 1&6-kestotetraose (1&6-KT) is formed when fructose is attached to the 6 position of the fructose unit bonded to the glucose and 1,6-kestotetraose (1,6-KT) when fructose is added to the 6 position of the terminal fructose (Fig. 1). Two major peaks were observed in the EIC traces for *m/z* 665 and 671 (Supplementary Figs. S2(a) and S2(b), see Supporting Information), corresponding to the *m/z* values of DP 4 fructans with no or one UL-$^{13}C_6$ fructose unit, respectively. The MS^2 spectra of both 1&6-KT and 1,6-KT showed mainly C and Z fragments (Fig. 2), as previously seen for inulin- and levan-type fructans.[17] Comparison of the MS^2 spectra of native 1&6-KT (Fig. 2(a)) and [UL-$^{13}C_6$ fru] 1&6-KT (Fig. 2(b)) indicated that only a small degree of B cleavage occurred. The major difference between the fragmentation patterns from 1&6-KT and 1,6-KT was the formation of a *m/z* 341 fragment. The *m/z* 341 fragment is released from 1,6-KT by a C_2 cleavage whereas it cannot be formed from 1&6-KT by a single bond cleavage (Fig. 1). Another point of differentiation between the two molecules is the production of the *m/z* 509 ion corresponding to a DP 3 fragment with one UL-labeled fructose. Labeled 6-kestotriose (*m/z* 509) is released from 1&6-KT (*m/z* 671) by a C_2 cleavage (Fig. 2(b)) but cannot be produced from 1,6-KT (*m/z* 671) (Fig. 2(c)) that can only form an unlabeled 1-kestotriose (*m/z* 503).

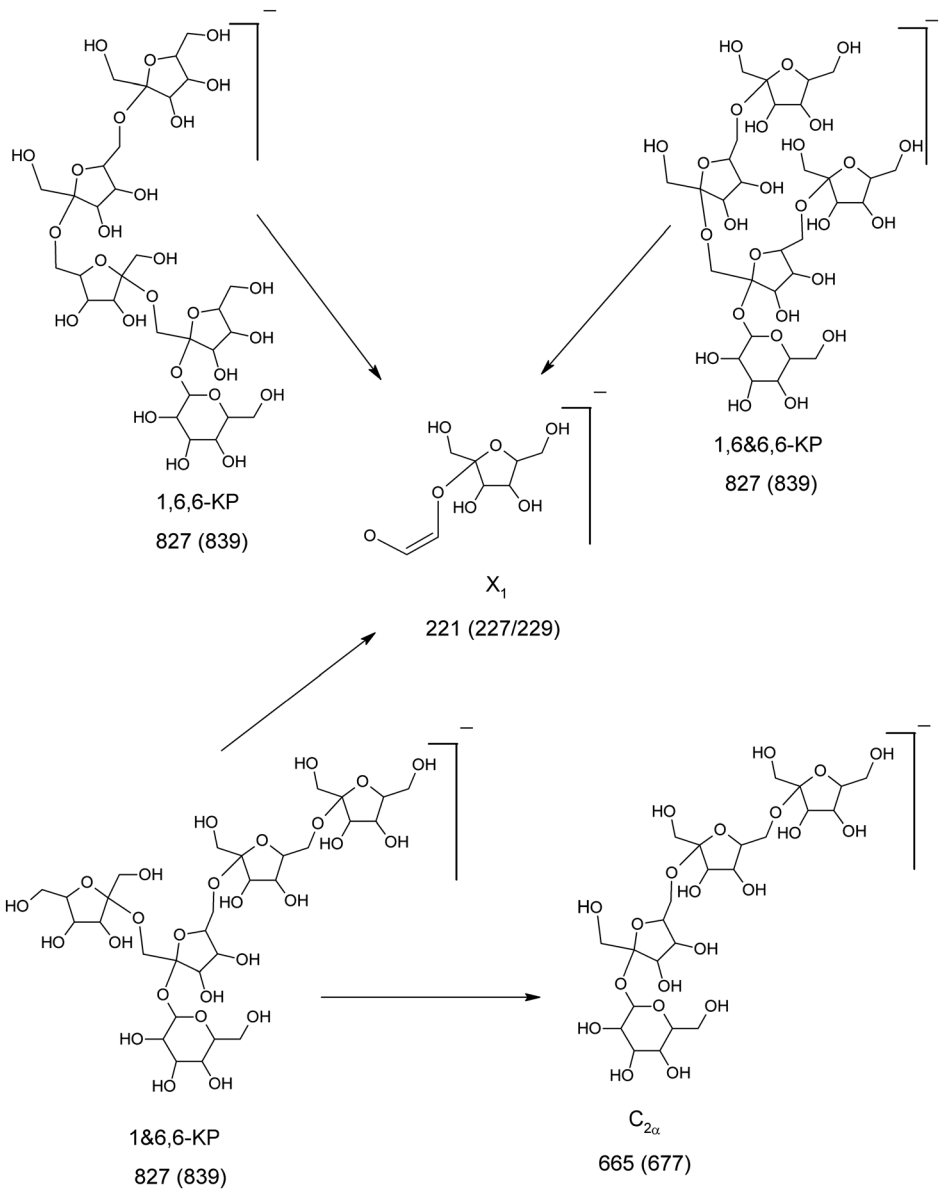

Figure 3. Molecular structures of the main DP 5 fructans formed by 6-SFT and the X_1 and $C_{2\alpha}$ fragment ions. The *m/z* values are indicated with between parentheses the *m/z* values of the corresponding [UL$^{13}C_6$ fru]-labeled species. X_1 (*m/z* 229) is released from two consecutively labeled fructose units whereas X_1 (*m/z* 227) is released from a labeled fructose bound to an unlabeled fructose. The $C_{2\alpha}$ fragment is only released from 1&6,6-KP.

In order to observe the low molecular mass fragments, MS^2 analysis was performed using HCD (Fig. 2(d)). The HCD spectrum of 1,6-KT (with one UL-labeled fructose unit) (*m/z* 671) contained an ion at *m/z* 227, corresponding to a labeled X_1 cross-ring cleavage product,[17] confirming that the labeled fructose is terminal and bonded β(2,6). Apart from 1&6-KT and 1,6-KT, three other DP 4 fructans were identified, although their concentrations in the reaction mixture were low (Supplementary Fig. S2(a), see Supporting Information). The identity of 6,6-KT and 1,1-KT (eluting at 1.9 and 8.7 min, respectively) was confirmed by comparing their MS^2 spectra with published data[17] and by use of authentic standards. As a result, the peak eluting at 5.5 min must have a 6G-kestotriose core, as all possible DP 4 fructans with a 1-kestotriose or 6-kestotriose core had already been identified. Only two structures could be formed by 6-SFT from 6G-kestotriose (i.e. 6G,6-KT and 6G&6-KT). The observation of the *m/z* 221 and 383 ions, corresponding to X_1 and X_2 cross-ring cleavage products, provides unequivocal evidence that 6G,6-KT is formed (Supplementary Fig. S3, see Supporting Information).

Fragmentation of DP 5 fructans

In theory, three kestopentaose (KP) fructan structures can be formed by 6-SFT starting from 1&6-KT or 1,6-KT, i.e. 1&6,6-KP, 1,6,6-KP and 1,6&6-KP (Fig. 3). Nevertheless, only two major peaks were observed in the EIC for DP 5 unlabeled fructans (*m/z* 827) or with two labeled fructose units (*m/z* 839) (Supplementary Fig. S4, see Supporting Information). The MS^2 spectrum of the first peak in the *m/z* 839 EIC contains a distinctive 677 fragment ion (Fig. 4(a)), indicating the presence of 1&6,6-KP. This fragment, containing two labeled fructose moieties, can be formed from 1&6,6-KP by a $C_{2\alpha}^{**}$ cleavage, but not from 1,6,6-KP or 1,6&6-KP. Yet, the same MS^2 spectrum also contains a clear 509 peak which cannot be formed from 1&6,6-KP by a single bond cleavage, this can only be explained by a C_2^* fragmentation of 1,6&6-KP. Hence, this suggests that two of the three possible DP 5 fructans are coeluting. The MS^2 spectrum of the second peak in the *m/z* 839 EIC (Fig. 4(b)) comprises a X_2^{**} fragment ion (*m/z* 395) corresponding to a cross-ring cleavage product containing two labeled fructose units that were β(2,6)-linked to the initial fructan structure.[17] Only 1&6,6-KP (*m/z* 839) and 1,6,6-KP (*m/z* 839) can form this fragment, but the absence of the 677 fragment indicates that this is the MS^2 spectrum of 1,6,6-KP. Further evidence for the identity of these structures was found in the HCD spectra. The HCD MS^2 spectrum of the first peak in the *m/z* 839 EIC (which corresponds to the KP with 2 UL-labeled fructose units) contains both fragment ions at *m/z* 227 and 229 (Fig. 4(c)). Both of these fragments originate from cross-ring cleavage from a terminal β(2,6)-linked fructosyl-fructose moiety (Fig. 3). The ion (*m/z* 227) results from the fragmentation of a structure containing an unlabeled fructose that is bound to a terminal labeled fructose unit whereas the second ion (*m/z* 229) originates from a structure containing two consecutively labeled fructose units. This observation once more shows that this MS^2 spectrum contains fragments from two different DP 5 structures as the original compounds contained only two UL-labeled fructose units (Fig. 3). The X_1^* ion (*m/z* 227) was released from doubly labeled 1,6&6-KP and the X_1^{**} ion (*m/z* 229) from doubly labeled 1&6,6-KP. The HCD MS^2 spectrum of the second peak in the EIC (*m/z* 839) (Fig. 4(c)) contains only the 229 (X_1^{**}) and 395 (X_2^{**}) cross-ring cleavage products, originating from a β(2,6)-linked fructosyl-β(2,6)-fructose moiety. This is in agreement with the previously assigned identity of 1,6,6-KP.

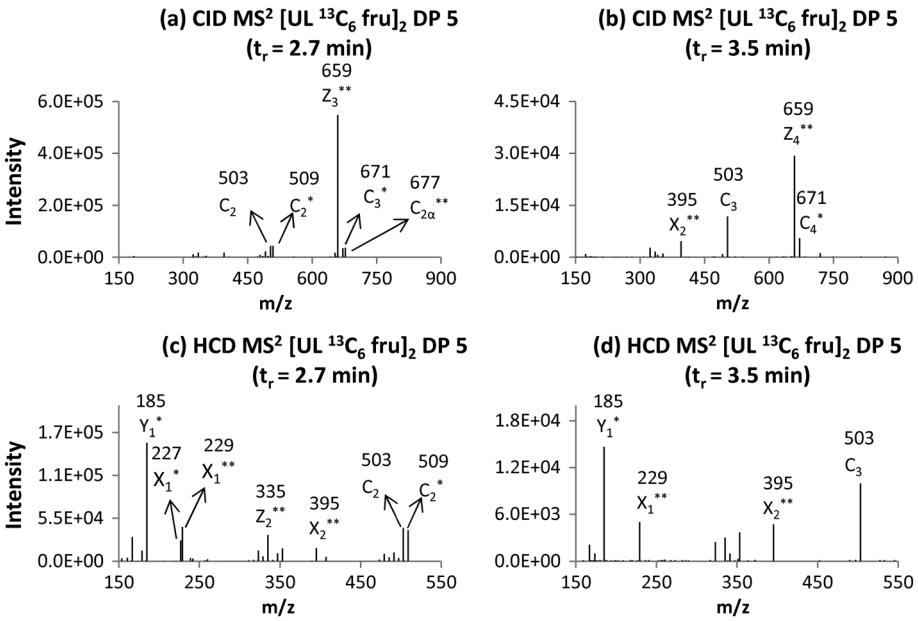

Figure 4. Negative electrospray MS^2 spectra of the main DP 5 fructans formed by 6-SFT. (a) CID MS^2 (*m/z* 839) of $[UL^{13}C_6\ fru]_2$ DP 5 fructans eluting at 2.7 min. (b) CID MS^2 (*m/z* 839) of $[UL^{13}C_6\ fru]_2$ DP 5 fructans eluting at 3.5 min. (c) *m/z* 150–550 region of HCD MS^2 (*m/z* 839) of $[UL^{13}C_6\ fru]_2$ DP 5 fructans eluting at 2.7 min. (d) *m/z* 150–550 region of HCD MS^2 (*m/z* 839) of $[UL^{13}C_6\ fru]_2$ DP 5 fructans eluting at 3.5 min.

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Fragmentation of DP 6 fructans

Four DP 6 fructan (or kestohexaose (KH)) structures can be formed by 6-SFT starting from the three DP 5 fructans identified in the previous paragraph (Figs. 5 and 6). Four major peaks were observed in the EIC for DP 6 fructans (*m/z* 989, Supplementary Fig. S5, see Supporting Information). The MS^2 spectra of the DP 6 fructans containing three UL- labeled fructose units (*m/z* 1007) were not informative because of high levels of interference from (1-kestotriose-1-kestotriose)$^-$ adducts. Therefore, MS^2 data were collected for 992, the *m/z* value of DP 6 fructans with three [1-$^{13}C_1$] fructose moieties, generated from the reaction mixture containing [1-$^{13}C_1$ fru] sucrose. The MS^2 spectra of the largest DP 6 peak (Fig. 7(a)) and the corresponding [1-$^{13}C_1$]$_3$-labeled species (Fig. 7(b)) contain X_2 and X_3 cross-ring cleavage products, and an X_1 cross-ring cleavage product was seen in the HCD spectrum (result not shown). These ions are indicative for three consecutively β(2,6)-linked fructose units and can only originate from either 1,6,6,6-KH or 1&6,6,6-KH. The

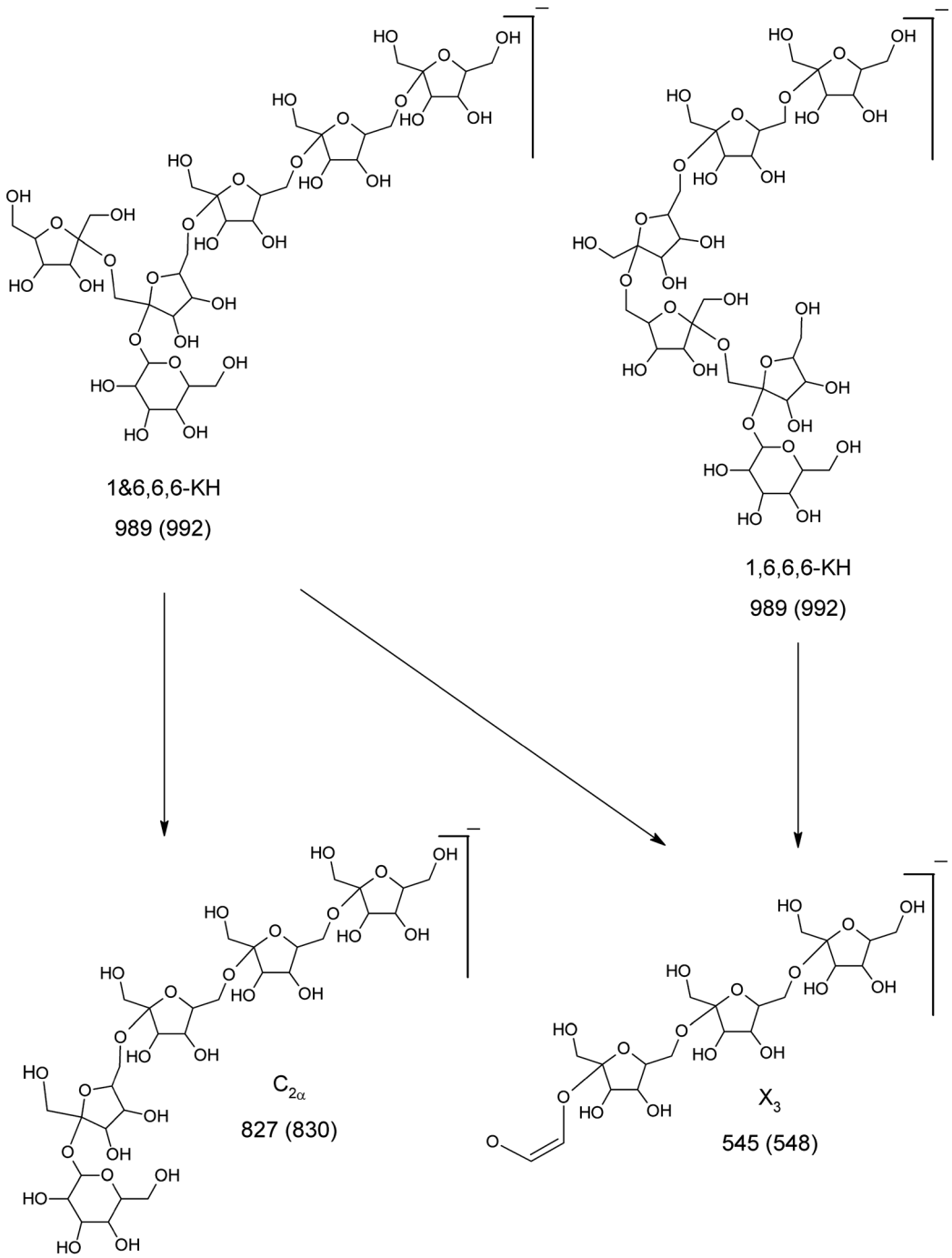

Figure 5. Molecular structures of 1,6,6,6-KH, 1&6,6,6-KH and the $C_{2\alpha}$ and X_3 fragment ions. The *m/z* values are indicated with between parentheses the *m/z* values of the corresponding [$^{13}C_1$ fru]-labeled species.

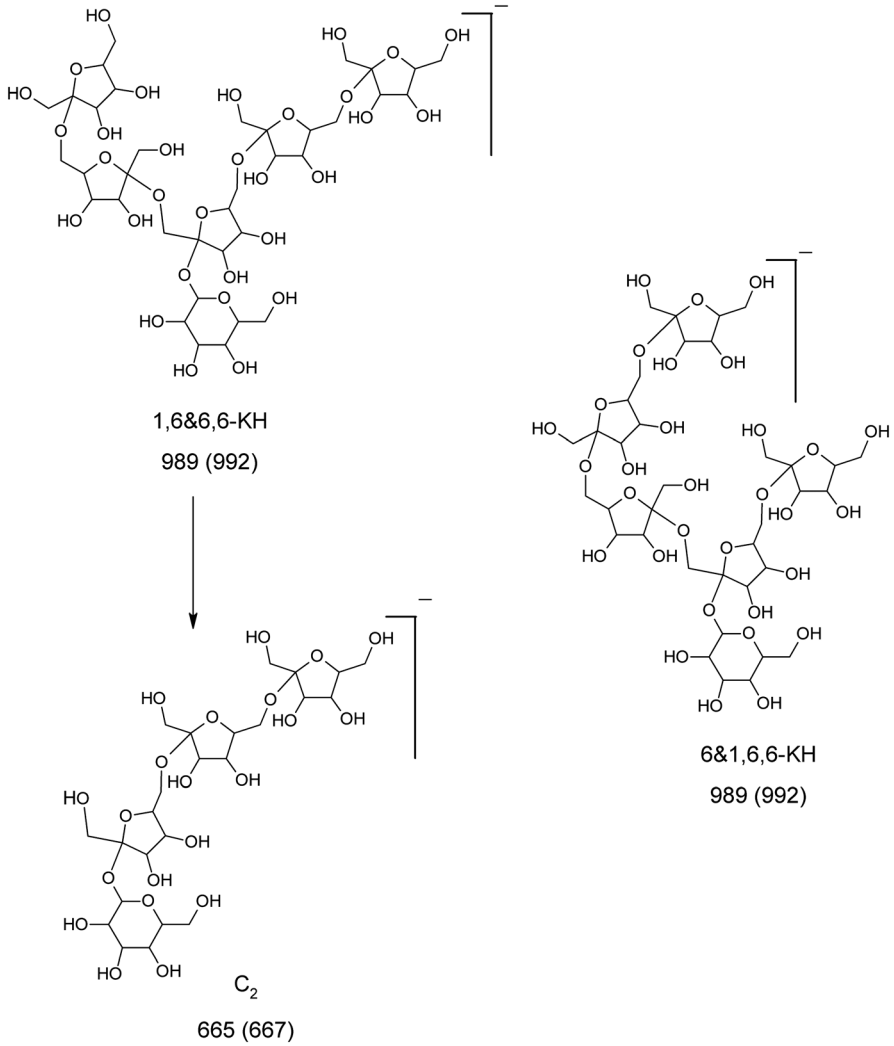

Figure 6. Molecular structures of 1,6&6,6-KH, 6&1,6,6-KH and the C_2 fragment formed from 1,6&6,6-KH. The *m/z* values are indicated with between parentheses the *m/z* values of the corresponding [$^{13}C_1$ fru]-labeled species.

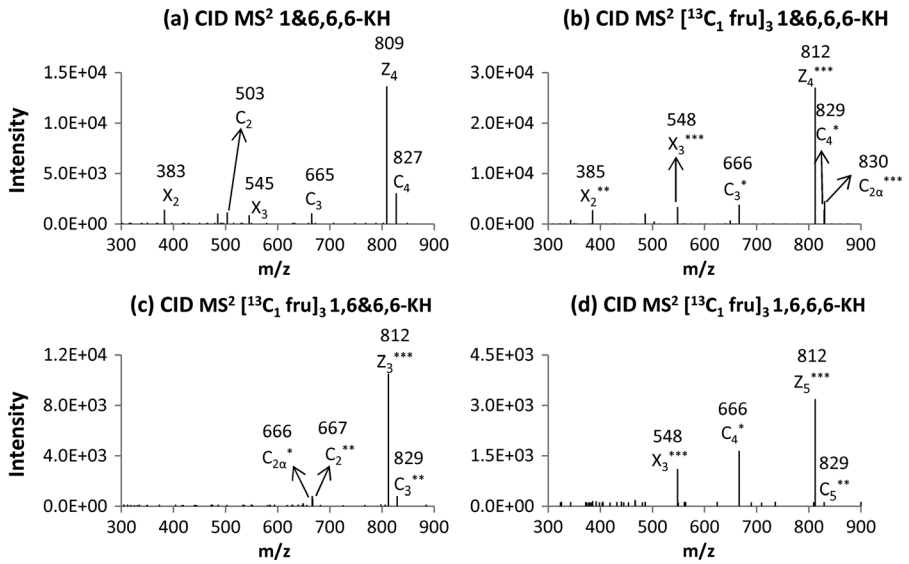

Figure 7. Negative electrospray MS^2 spectra of the main DP 6 fructans formed by 6-SFT. (a) CID MS^2 (*m/z* 989) of unlabeled 1&6,6,6-KH. (b) CID MS^2 (*m/z* 992) of [$^{13}C_1$ fru]$_3$ 1&6,6,6-KH. (c) CID MS^2 of (*m/z* 992) [$^{13}C_1$ fru]$_3$ 1,6&6,6-KH. (d) CID MS^2 of (*m/z* 992) [$^{13}C_1$ fru]$_3$ 1,6,6,6-KH.

formation of the $C_{2\alpha}^{***}$ (*m/z* 830) fragment in the MS^2 spectrum of the [1-$^{13}C_1$]$_3$-labeled species excludes the first possibility (Fig. 5) and, consequently, it can be inferred that 1&6,6,6-KH was the major DP 6 fructan formed by 6-SFT. The MS^2 spectrum of the second peak (peak B in Supplementary Fig. S5(b), see Supporting Information) in the EIC for tri-labeled DP 6 fructans contained a distinctive *m/z* 667 ion (Fig. 7(c)). This fragment may result from a C_2^{**} cleavage of 1,6&6,6-KH (*m/z* 992) but cannot be formed from the three other possible DP 6 fructan structures (Figs. 5 and 6) when only one bond is cleaved. In the MS^2 spectrum of the last DP 6 fructan (peak D in Supplementary Fig. S5(b), see Supporting Information), an X_3^{***} fragment (*m/z* 548) is observed, indicating that three labeled fructose units were β(2,6)-linked to the original compound. This observation together with the absence of a *m/z* 830 fragment demonstrates that the spectrum belongs to the unbranched 1,6,6,6-KH (Fig. 5). The MS^2 spectrum of the compound eluting first in the DP 6 EIC (peak A in Supplementary Fig. S5(b), see Supporting Information) contained a Z^{***} fragment (*m/z* 812), together with a *m/z* 811 fragment (results not shown). The latter can be formed from 6&1,6,6-KH, the only remaining DP 6 fructan formed by 6-SFT (Fig. 6). Unfortunately, this conclusion could not be confirmed by the observation of distinctive fragments from the low *m/z* region because the ion current was too low to obtain reliable MS^2 spectra.

CONCLUSIONS

It can be concluded that incubation of 1-kestotriose with ^{13}C-labeled sucrose and *P. terminalis* 6-SFT made it possible to elucidate the fragmentation pattern of all DP 4, 5 and 6 graminan-type fructans produced. Using the same approach, based on LC/MS analysis of ^{13}C-labeled fructan oligomers, structural characterization of higher DP fructans should be feasible as long as the ion current and hence signal-to-noise ratio is sufficiently high. In this case, higher order MS^n ($n > 2$) analysis may be required for full structural elucidation since the number of theoretically possible isomers increases rapidly with increasing DP. In the present study, the major peaks in the DP 5 and DP 6 EICs contained 1&6,6-KP and 1&6,6,6-KH, respectively. These structures both have a 1&6-KT core to which one or two fructose units are attached via a β(2,6) linkage. This suggests that *P. terminalis* 6-SFT prefers to extend the existing branch above creating a new branch. Analysis of the DP 3 fructans in the reaction mixture with UL-^{13}C indicated that *P. terminalis* 6-SFT has no 6G-FFT activity but does have 6-SST and minor 1-SST side activities.

The method described above based on the MS^2 fragmentation routes enables not only the LC/MS-based characterization of fructan-metabolizing enzymes, but also a rapid screening of large sample sets for the presence of branched, graminan-type fructans. To this end, the presence of fructan oligosaccharides could be verified by MS^1 analysis. Next, their structures can be determined based on their LC elution order and by performing MS^2 analysis for the corresponding masses (summarized in Table 1). An interesting application is the study of the effects of food processing on this type of fructans. Alternatively, the effect of their fermentation by the colon microbiota can be investigated by LC/MS analysis of partially fermented fructans obtained from either *in vitro* or *in vivo* fermentation experiments. A third application is the study of fructan metabolism in plants by the structural characterization of fructans extracted from kernels or derived tissues from different phases of development as described for immature wheat grains.[23]

The EICs of the DP 3, 4, 5 and 6 of both native and ^{13}C-labeled 6-SFT reaction products and DP 3 and DP 4 fructan standards are added as Supporting Information together with the negative electrospray MS^2 spectra of native and UL-labeled 6G,6-kestotetraose. In addition, HPAEC profiles showing the gradual formation of branched fructans are included. Finally, a summary of the 6-SFT reaction products and their ion intensities is provided.

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